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PROTEINS ENCODED BY RAS ONCOGENES STIMULATE OR INHIBIT PHOSPHORYLATION OF SPECIFIC MITOCHONDRIAL MEMBRANE PROTEINS

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We have examined the effects of a series of purified p21 proteins encoded by ras oncogenes and synthesized in E.coli via recombinant DNA methods, on the phosphorylation of proteins associated with isolated rat liver mitochondria. We find that these proteins markedly enhance the phosphorylation of a 36KD protein and inhibit phosphorylation of a 17KD protein. The phosphorylated residues on the 36KD protein are hydrolyzed by mild acid, suggesting that they involve phosphoamide bonds. These results suggest that p21 proteins may play a role in vivo by altering the phosphorylation of certain proteins.

The ras oncogenes transform cells through the action of a family of 21 kilodalton (KD) proteins termed p21 (1,2). These proteins bind guanine nucleotides and have GTPase activity (3-5). They also have extensive sequence homology with α-subunits of several regulatory guanine nucleotide binding proteins (G-proteins) (6-8). The precise biochemical functions of the ras p21 proteins in normal and transformed cells are not known, although there are suggestions that they might play a role as a α-subunit in a G-protein (for review see ref. 9). In the present paper we describe an in vitro system employing isolated rat liver mitochondria which leads to the phosphorylation of several proteins. We demonstrate that the addition of purified ras proteins, synthesized in E. coli by recombinant DNA methods, markedly influences the phosphorylation of two of these proteins.

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MATERIALS AND METHODS

Female Sprague-Dawley rats (90-150 days old) were anesthetized with diethylether, sacrificied by cervical dislocation and the livers rapidly excised. Mitochondria were isolated at 4°C by differential centrifugation in a pH 7.4 medium containing: 0.21 M D-mannitol, 1 mM HEPES, 0.1 mM EGTA and 1 mg/ml defatted serum albumin, essentially as described by Greenawalt (10). Phosphorylation was performed at 20°C in 0.1 ml of standard reaction mixture that contained: 0.21 M D-mannitol, 0.07 M sucrose, 1 mM HEPES at pH 7.4, 2.5 mM $MgCl_2$, 10 mM potassium succinate, 2 ug/ml rotenone, 2 ug/ml oligomycin (Sigma), 5 ug/ml carboxyatractyloside (Sigma), and 100-200 ug/ml mitochondrial protein. Reactions were started by the addition of 5-10 uCi of [γ-^{32}P]ATP (>5000 Ci/mmole, Amersham) and terminated by the addition of 0.02 ml of 20% SDS followed by 0.03 ml sample buffer, which contained: 10% (v/v) glycerol, 1 g/l bromphenol blue, 20% (v/v) β-mercaptoethanol, amd 0.06 M Tris HCl pH 6.8. Samples were boiled for 3 minutes and then analyzed by discontinous SDS-polyacrylamide gel electrophoresis. The stacking gel was polymerized from 3% acrylamide, 0.15% bisacrylamide in 0.1% (w/v) SDS and 0.125 M Tris HCl pH 6.8. The resolving gel was 10% acrylamide with an acrylamide/bisacrylamide ratio 20:1 in 0.1% SDS and 0.375 M Tris HCl pH 8.3. The electrophoresis buffer contained:0.025 M Tris HCl pH 8.3, 0.192 M glycine and 0.1% SDS. Molecular weight markers (BRL) were run on the same gel. Prior to autoradiography, gels were fixed in 10% 2-propanol, containing 10 mM ATP and 10 mM sodium pyrophosphate. Gels were autoradiographed using Kodak X-Omat AR film, with 1-3 days exposure at -70°C. In some experiments autoradiographed gels were treated overnight with 1:1 glacial acetic acid: methanol solution (each at 15% v/v) and autoradiographed again to examine the profile of acid-stable phosphoproteins.

RESULTS

When freshly isolated rat liver mitochondria were incubated aerobically for two minutes in the presence of succinate, rotenone (to block NADH-linked electron transport), oligomycin (to inhibit the formation of nonradioactive ATP), carboxyatractyloside (to block uptake of the radioactive ATP into mitochondria) and [γ-^{32}P]ATP, four proteins were reproducibly labeled with ^{32}P, which migrated in SDS-polyacrylamide gels to positions that corresponded to 107, 70, 36 and 17KD (Figure 1A, lane 1). The 107, 70 and 36KD radioactive bands shown in Figure 1A were not seen, but the 17KD band persisted, when the same gel was treated overnight with 15% acetic acid prior to autoradiography (Figure 1B, lane 1). The 17KD band was usually

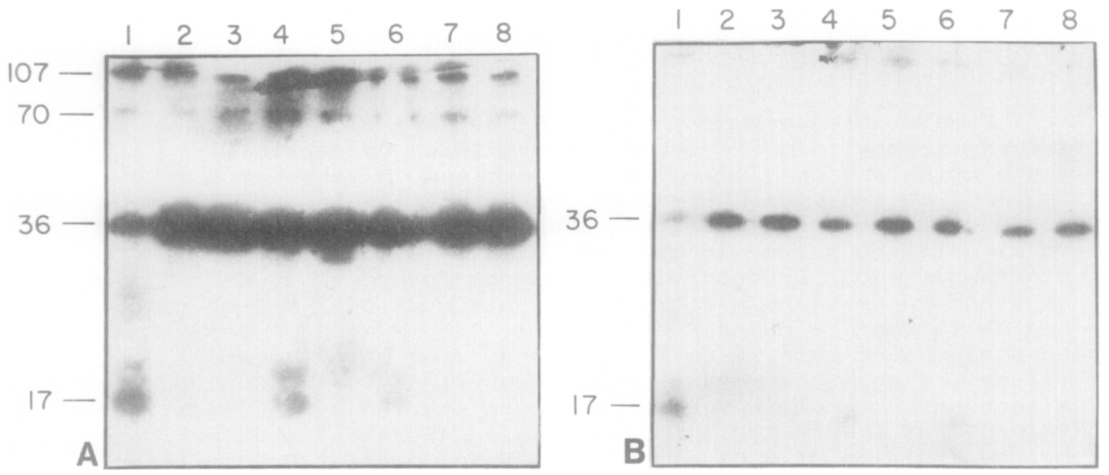

FIGURE 1. Effects of various ras p21 proteins on phosphorylation of mitochondrial proteins. Panel A. Lane 1, mitochondria incubated alone. Lanes 2-8, mitochondria incubated with 50 ug/ml of the following ras p21 proteins: lane 2, RAS1,; lane 3, RAS2; lane 4, EJ; lane 5, EJ/v-Ha-ras; lane 6, EC/leu-61; lane 7, EC; lane 8, EC/v-Ha-ras. Molecular weights in kilodaltons of the major phosphorylated proteins are indicated at the left. Panel B. Same as Panel A, but autoradiography was done after acid-treatment of the gel.

broad and in some experiments was resolved into a doublet. Both components of the doublet responded similarly to the addition of p21 proteins (see below).

Phosphorylation of mitochondrial proteins was assayed in the absence and presence of seven different p21 proteins, each of which had been expressed in E. coli and purified to homogeneity (11-14): 1). RAS1, viral Harvey ras p21 with Arg-12, Thr-59, Gln-61 (14), 2) RAS2, viral Harvey ras p21 with Arg-12, Thr-59, Gln-61 and no amino-terminal fusion sequence (Stein, R.B., unpublished studies), 3) EJ, with Val-12, Ala-59, Gln-61 (12), a transforming p21 from a human bladder tumor (15), 4) EJ/v-Ha-ras, EJ but with Thr-59, instead of Ala-59 (12), 5) EC, with Gly-12, Ala-59, Gln-61, the normal human Harvey ras oncogene product (13), 6) EC/v-Ha-ras, EC but with Thr-59, instead of Ala-25 (12), and 7) EC/Leu-61, EC but with Leu-61, instead of Gln-61 (16), a transforming p21 from a human lung carcinoma (17). Six of the preceding seven p21 proteins were expressed in E. coli as fusion

proteins with an amino-terminal fusion sequence extending from -19 to 0; RAS2 p21 does not have an amino-terminal fusion sequence. All seven purified p21 proteins markedly enhanced the extent of phosphorylation of the mitochondrial 36KD protein and, but for EJ p21 protein, completely inhibited phosphorylation of the mitochondrial 17KD protein (Figure 1A). Figure 1B demonstrates that most of the ^{32}P incorporated into the 36KD protein, in the absence and in the presence of different p21 proteins, was removed by mild acid treatment, suggesting that the phosphorylation of this protein involved phosphoramidates, since they are known to be acid labile (18). We saw no consistent effect of p21 proteins on phosphorylation of the mitochondrial 107 and 70KD proteins. Thus, the remainder of this paper focuses on the 36 and 17KD proteins.

Our preparations of p21 proteins contained GDP (11), which binds to the p21 proteins with a dissociation constant of about $10^{8}M^{-1}$ (19). At the micromolar concentrations of p21 proteins employed in our experiments 5-10% of the bound GDP would be dissociated from the protein at equilibrium. We investigated, therefore, possible effects of GDP itself on the phosphorylation of the 17 and 36KD mitochondrial proteins. Figure 2A demonstrates that free GDP (in the range 50-500 nM) inhibited phosphorylation of the 17 KD protein to a greater extent than an amount of EC p21 protein that contains an approximately equivalent amount of GDP. Thus, it is possible that the effect of added p21 proteins on phosphorylation of the 17KD protein may be due, at least in part, to the GDP bound to this protein, which could dissociate in the incubation system. Although very low concentrations of free GDP (50-250 nM) stimulated the phosphorylation of the 36KD protein, higher concentrations were inhibitory (Figure 2B). Both the EC and EJ p21 proteins

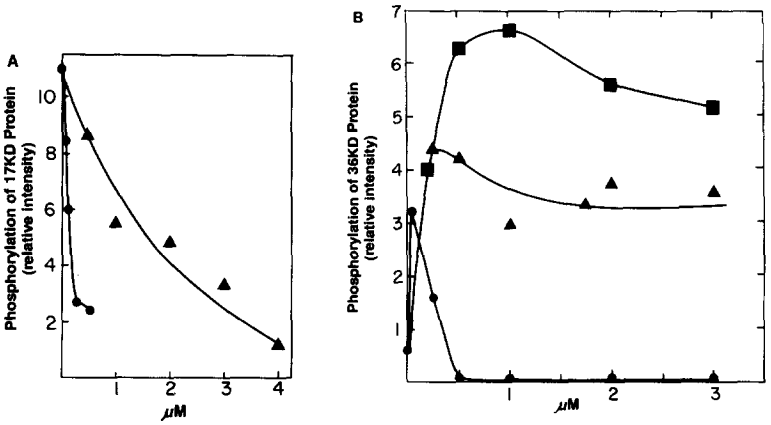

FIGURE 2. Effects of GDP and purified ras p21 proteins on phosphorylation of the mitochondrial 17KD (panel A) and 36KD (panel B) proteins. Experiments were performed as described in MATERIALS AND METHODS in the presence of indicated amounts of GDP or ras p21 proteins, both expressed on a molar basis. The relative intensities of the 36 and 17KD bands on the autoradiographs were measured with a Chromoscan 3 Joyce Loeble densitometer. —●— , effect of GDP. —▲— , effect of EC p21 protein; —■— , effect of EJ p21 protein.

stimulated the phosphorylation of this protein to a higher level than that obtained with free GDP, and this stimulation persisted even at high concentrations of these proteins (Figure 2B). Thus, the p21 proteins are more effective stimulators of 36KD protein phosphorylation than free GDP, although our studies do not exclude a role for protein-bound GDP in this process.

DISCUSSION

This study demonstrates that seven different bacterially expressed p21 proteins markedly affect the phosphorylation of two mitochondrial proteins. The phosphorylation of a 17KD protein is inhibited, while the phosphorylation of a 36KD protein is enhanced. These reciprocal effects (Figure 1) provide evidence that these results are not due to contamination of the p21 proteins with a bacterial protein kinase or phosphoprotein phosphatase. The inhibitory effects of p21 proteins on phosphorylation of the 17KD protein may be due, at least in part, to their content of bound GDP, but it is unlikely that GDP alone accounts for the stimulatory effects on phosphorylation of the

36KD protein (Figure 2). Nor is it likely that the N-terminal fusion sequences present in six of the seven p21 proteins employed in this study are responsible for the effects we have observed, since these effects were seen with both RAS1 p21 protein and RAS2 p21 protein (Figure 1), which are the same proteins except that the former contains a fusion sequence but the latter does not. In the present studies we did not observe a consistent difference between normal and transforming p21 proteins in terms of their effects on phosphorylation of the 36 and 17KD proteins. This result may be relevant to the finding that both point mutations in the ras genes and overexpression of the normal p21 protein can cause transformation of cultured cells (1,2). Studies are in progress to determine whether a more purified in vitro system will discriminate between the normal and mutated ras p21 proteins.

The mitochondrial incubation assays used in the present studies contained carboxyatractyloside, an inhibitor of ATP uptake by mitochondria. Since under these conditions we obtained phosphorylation of the 17 and 36KD proteins it appears that these proteins are associated with the outer face of the inner mitochondrial membrane or with the outer mitochondrial membrane. Indeed, studies in progress indicate that these proteins can be released from the outer mitochondrial membrane by incubation of isolated mitochondria with 10 mM glucose-6-phosphate for 30 minutes at 20°C (unpublished studies). Since ras p21 proteins are known to be associated with the inner surface of the plasma membrane (20) it is somewhat unexpected that they affect phosphorylation of mitochondrial proteins in our subcellular system. It is known that mitochondrial membranes contain a guanine nucleotide binding protein and that in brown adipose tissue this protein regulates mitochondrial thermogenesis (21).

perhaps, ras p21 proteins share homology with the α-subunit of a mitochondrial G protein that influences the phosphorylation of specific mitochondrial proteins. We are currently examining this possibility and also studying whether the ras p21 proteins have a similar influence on the phosphorylation of proteins associated with the plasma membrane.

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